PATENT
Attorney Docket: GC636-2

Enhanced Secretion of a Polypeptide by a Microorganism

5

Inventor:
Marc Kolkman

CROSS-REFERENCE TO RELATED APPLICATIONS

10   Pursuant to 35 U.S.C. §119(e), the present application claims benefit of and priority to USSN 60/239,531, entitled "Enhanced Secretion of a Polypeptide by a Microorganism", filed October 10, 2000, by Marc Kolkman.

FIELD OF THE INVENTION

15   This invention relates to the production and secretion of a selected polypetide. More particularly, the present invention provides for the enhanced secretion of a selected polypeptide by a microorganism, such as a *Bacillus* species.

BACKGROUND OF THE INVENTION

20   Eubacteria export numerous proteins across the plasma membrane into either the periplasmic space (Gram-negative species), or the growth medium (Gram-positive species). The Gram-positive eubacterium *Bacillus subtilis* and, in particular, its close relatives *Bacillus amyloliquefaciens* and *Bacillus licheniformis* are well known for their high capacity to secrete proteins 25   (at gram per liter concentrations) into the medium. This property, which allows the efficient separation of (secreted) proteins from the bulk cytoplasmic protein complement, has led to the commercial exploitation of the latter bacilli as important "cell factories." Despite their high capacity to secrete proteins of Gram-positive origin, the secretion of recombinant proteins from Gram-
30   negative eubacterial or eukaryotic origin by *Bacillus* species is often inefficient.

General strategies for the secretion of heterologous proteins by bacilli are based on the in-frame fusion of the respective protein with an amino-terminal signal peptide that directs this protein into a secretion pathway, for example the Sec-dependent secretory pathway. Upon translocation across

5   the membrane, the signal peptide is removed by a signal peptidase, which is a prerequisite for the release of the translocated protein from the membrane, and its secretion into the medium.

Proteolysis in bacteria serves to rid the cell of abnormal, and misfolded proteins. A unique mechanism for the destruction of abnormal proteins

10   resulting from abortive termination of translation is provided by the SsrA-mediated tagging and degradation system (for a recent review, see Karzai et al. (2000). *The SsrA-SmpB system for protein tagging, directed degradation and ribosome rescue.* Nat. Struct. Biol. 7:449-455.). SsrA, also called 10Sa RNA or tmRNA is a highly conserved RNA molecule in eubacteria. It is a

15   unique molecule that can act as both a tRNA and an mRNA in a process referred to as *trans*-translation (Atkins et al. 1996. A case for *trans* translation. Nature 379:769-771, Jentsch 1996. When proteins receive deadly messages at birth. Science 271:955-956, Keiler et al. 1996. Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger

20   RNA. Science 271:990-993). This mechanism provides the cell a way to release ribosomes that are stalled on untranslatable mRNAs, e.g. mRNAs lacking in-frame stop codons. In the model for SsrA action, SsrA charged with alanine enters the A site of a stalled ribosome, mimicking a tRNA. The alanine is added to the uncompleted polypeptide chain; and then, serving as

25   an mRNA, SsrA provides a short reading frame followed by a stop codon as a template to add a short peptide to the nascent polypeptide before translation terminates and a tagged protein is released. The peptide tag (encoded by SsrA) functions as a proteolytic degradation signal, and in *Escherichia coli* four proteases have been identified that degrade proteins tagged by SsrA.

30   ClpXP, ClpAP, and FtsH (HflB) degrade SsrA tagged proteins in the

GC636-2

cytoplasm (Gottesman et al. 1998. The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. Genes Dev. 12:1338-1347, Herman et al. 1998. Degradation of carboxy-terminal-tagged cytoplasmic proteins by the *Escherichia coli* protease

5   HflB (FtsH). Genes Dev. 12:1348-1355), while SsrA tagged proteins with signal peptides that are exported to the periplasm of *E. coli* are degraded by Tsp (Prc) protease (Keiler et al. 1996).

Protein production and secretion from *Bacillus* species is a major production tool with a market of over $1 billion per year. However, proteolysis

10   of proteins by endogenous proteases diminishes the production capability of these microorganisms. Thus, it would be beneficial to have an mechanism for the enhanced production and secretion proteins. The present invention provides such an advantage by changing the nonpolar C-terminus of a protein by adding charged, polar residues (or by replacing amino acids), so that the

15   proteins are protected against the bacillus proteases that degrade SsrA-tagged proteins.

SUMMARY OF THE INVENTION

Provided herein are methods for the enhanced production of peptides in a host cell.

20   In one aspect of the invention, the present invention provides methods for increasing secretion of proteins from host microorganisms. In one embodiment of the present invention, the protein is homologous or naturally occurring in the host microorganism. In another embodiment of the present invention, the protein is heterologous to the host microorganism. Accordingly,

25   the present invention provides a method for increasing secretion of a protein in a host cell using an expression vector comprising nucleic acid sequence encoding a protein of interest wherein said nucleic acid sequence is under the control of expression signals capable of expressing said protein of interest in a host microorganism; introducing the expression vector into a host

30   microorganism capable of expressing said protein and culturing said

GC636-2